TRƯỜNG ĐẠI HỌC SƯ PHẠM TP HỒ CHÍ MINH
TẠP CHÍ KHOA HỌC
KHOA HỌC TỰ NHIÊN VÀ CÔNG NGHỆ
ISSN: 1859-3100 Tập 14, Số 9 (2017): 143-151

HO CHI MINH CITY UNIVERSITY OF EDUCATION
JOURNAL OF SCIENCE
NATURAL SCIENCES AND TECHNOLOGY
Vol. 14, No. 9 (2017): 143-151

Email: tapchikhoahoc@hcmue.edu.vn; Website: http://tckh.hcmue.edu.vn

ISOLATION OF CALCITE PRECIPITATION BACTERIA TO IMPROVE THE STRENGTH OF CONCRETE

Le Quynh Loan[1], Nguyen Luong Hieu Hoa[2], Tran Thi My Ngoc[1], Duong Thi Phung Cac[3], Nguyen Hoang Dung[1,2*]

[1]Institute of Tropical Biology - VAST
[2]NTT Hitech Institute, Nguyen Tat Thanh University
[3]Biotechnology Center of Ho Chi Minh City

Received: 17/8/2017; Revised: 25/8/2017; Accepted: 23/9/2017

ABSTRACT

Concretes are the second most consumed material on earth. However, it is susceptible to micro crack formation and has pores in it. The repairing cracks in concrete require high cost and labor and traditional repair system are chemical based, expensive and lead to environmental and health hazards. In this study, the calcite precipitation bacteria were investigated. Bacterial strains were isolated from cement samples and were tested for urease activities, potential to form endospore and calcite precipitation. The results showed that four candidates were isolated with high urease activities and calcite precipitation. Among them, the bacterial isolate N1 could precipitate 0.5 g $CaCO_3$ per liter. By using 16S rDNA sequencing, this strain was identified as Bacillus thuringiensis. The ability of this strain to tolerate the extreme environment of cement, it could be used in remediating the cracks and fissures in various building or concrete structure.

Keywords: calcite precipitation, bacteria, concrete, isolation.

TÓM TẮT

Phân lập vi khuẩn có khả năng tạo kết tủa Calcite hướng đến mục đích tăng độ bền của bê tông

Bê tông là vật liệu được tiêu thụ nhiều thứ hai trên thế giới. Tuy nhiên, theo thời gian sử dụng thì vật liệu này lại dễ hình thành các vết nứt và các lỗ li ti trên bề mặt. Việc sửa chữa các vết nứt trên bê tông đòi hỏi chi phí và lao động cao; phương pháp truyền thống được sử dụng là dựa vào hóa chất, phương pháp này đắt tiền và ảnh hưởng các vấn đề môi trường và sức khỏe. Trong nghiên cứu này, chúng tôi hướng đến khảo sát các vi khuẩn tạo calcite. Vi khuẩn được phân lập từ các mẫu xi măng và khảo sát hoạt tính urease, khả năng tạo bào tử và khả năng hình thành kết tủa calcite. Trong số các chủng vi khuẩn đã phân lập, chủng N1 có thể tạo calcite với sản lượng 0.5g/L. Bằng phương pháp giải trình tự vùng gen 16S rDNA, chủng N1 đã được định danh thuộc loài Bacillus thurigiensis. Chủng vi khuẩn này cho thấy có khả năng tồn tại lâu dài trong môi trường khắc nghiệt như xi măng, và có tiềm năng được sử dụng để làm liền các vết nứt trong công trình xây dựng hoặc các cấu trúc bê tông khác.

Từ khóa: kết tủa calcite, vi khuẩn, bê tông, phân lập.

[*] Email: *dung0018034@yahoo.com*

1. Introduction

Calcite precipitation bacteria have been studied mainly for application in the fields of surface protection of natural stone, crack remediation in concrete and soil improvement [1]. Concrete is the second most consumed material on earth, however, crack formation is a phenomenon that can hardly be complete avoided. The repairing cracks in concrete require high cost and labor and traditional repair system are chemical based, expensive and lead to environmental and health hazards [2]. In recent years, the application of bacteria for construction purpose has become a topic of research worldwide. It is expected that the calcite precipitation bacteria could apply in crack healing concrete and will result in a more durable that could replace the traditional concrete constructions.

It has been known that microorganisms play an important role in promoting natural calcite precipitation. Calcite precipitation is a general phenomenon in the bacterial world and under suitable conditions most bacteria are able to precipitate calcite crystals. There exist in nature four different processes that can be harnessed for calcite precipitation: Carbonic andydrate, sulphate reduction, nitrate reduction, and urea hydrolysis [3,4]. Most of the studied bacteria on calcite precipitation are base on urea hydrolysis process, as it is controlled by urease enzyme [5,6]. Urease (urea amidohydrolase: EC 3.5.1.5) is an enzyme that hydrolyzes urea into carbonate and ammonia ions, as shown in reaction (1).

$$(NH_2)_2CO + 2H_2O \rightarrow CO_3^{2-} + 2NH_3 \quad (1)$$

Through the urease reaction, ammonia will increase the pH which favors the calcite formation [6]; carbonate ions are release into environment, and then could bind with calcium to form calcium carbonate and its metastable polymorph, calcite, as shown in reaction (2).

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad (2)$$

Calcite-precipitation bacteria has been reports in various environments that include limestone, caves, soils and seawater [7]. Many urease producing bacteria have been investigated, including *Aerobacter aerogenes, B. megaterium, B. subtilis, Bacillus* sp. CR2, *B. thuringiensis, D. halophila, Halmonas eurihalina, Helicobacter pylori, Kocuria flava* CR1, *L. sphaericus* CH5, *Methylocystis parvum, Myxococcus xanthus, Proteus mirabilis, Pseudomonas denitrificans, Spoloactobacillus* sp., *Sporosarcina ginsengisoli and Sporosarcina pasteurii* [8-14]

The purpose of this study was to isolate and characterize the calcite precipitation bacteria from cement sample, and selecting strain to be utilized in biotechnological application. This is the first survey for further investigation and application about crack healing concrete at Vietnam.

2. Materials and methods

2.1. Enrichment of Sample and Bacteria Isolation

The Cement samples were collected from the commercial bags and placed in sterilized bottle. To enrich the cement samples for calcite precipitation bacteria, 1g of cement was inoculated into 50 ml nutrient broth (HiMedia, India) (pH 8.0) containing 2% urea, and incubates at 37ºC for 120h under shaking condition (130 rpm). Then the sample was serially diluted and plated on carbonate precipitation agar containing urea (20 g/l), $NaHCO_3$ (2.12 g/l), NH_4Cl (10g/l), Nutrient broth (3 g/l), $CaCl_2.2H_2O$ (25 g/l). Incubation was done at 30ºC. Colonies were assessed every 5 days and selected as positive based on visual crystal formation within 10 days. Positive isolates were purified through repetitive dilution and plating as described earlier.

2.2. Crystals Identification

The isolated strains were inoculated on B4 liquid medium (4g/l yeast extract, 2.5 g/l calcium acetate, and 10 g/l glucose) [15], incubated at 30ºC for 2 weeks and were daily examined for presence of crystals. To identify whether the crystals formed by bacteria were calcite, a simple test with diluted hydrochloric acid (HCl) was carried out. If calcite was present, it will dissolve in the presence of HCl, with the release of tiny gas bubbles of carbonate.

2.3. Calcium Carbonate Precipitation and Collection

For calcium carbonate precipitation and collection, bacteria were grown aerobically in 100 mL of liquid calcium carbonate precipitation media and incubated at 30 °C for 60 h. The control consisted of uninoculated liquid calcium carbonate precipitation medium. After the incubation, the whole culture was centrifuged at 10,000 g for 1 min. The pellet, which included calcium carbonate precipitate and the bacteria cells, was resuspended in 50 mL TE buffer (10 mM Tris, 1 mM EDTA pH 8.5). Lysozyme was added at a final concentration of 1 mg/mL and the cell suspension was incubated at 37 °C for 1 h to digest the bacteria cell wall. The cell debris was removed by centrifugation and the pellet was washed with sterile distilled water (pH 8.5), then air dried at 37 °C for 24 h. The pellet was weighed to estimate the amounts of carbonate crystals precipitated by bacteria [16].

2.4. Microscope Phenotypic Characterization

Gram staining and endospore staining is the fundamental to the phenotypic characterization of bacteria which is a differential staining procedure, based on the bacterial ability to retain the color of the stains used in the procedure.

2.5. Urease Activity

All the isolates were tested for urease activity. This was done by inoculating test broth with viable liquid cultures (20g/l urea, 9.1 g/l Monopotassium phosphate, 9.5 g/l disodium phosphate, 0.1 g/l yeast extract, 0.01 g/l phenol red). Positive strains turning the indicator phenol red from its original orange yellow color to bright pink [17].

2.6. DNA Extract, PCR Amplification and Sequencing

Bacterial genomic DNA was extracted from pure culture with the fast spin kit (Invitrogen) following the manufacturer's instructions. Amplification of 16S rRNA gene was performed in 50 μL of reaction mixture containing 0.25 mM each primer of 27f (5′-GTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACCTTGTTACGACTT-3′), 0.2 mM dNTP, 1.5 mM MgCl2, 5 μL of Taq buffer, and 5 U Taq DNA polymerase (NEB, USA), 10–20 ng template DNA. PCR was then performed on a thermalcycler under the following conditions: 95 °C for 5 min, 35 cycles of 50 s at 95 °C, 50 s at 45 °C and 1.5 min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products were visualized on an agarose gel, and the bands with the corrected size were excised and purified using the ISOLATE II gel purification protocol (Biolink, Singapore). The partial 16S rRNA fragment was then sequenced and analyzed by using the Blastx software (BLAST) (National Center for Biotechnology Information) [16].

3. Results and discustion

3.1. Isolation of Calcite Precipitation Bacteria from Cement Samples

The commercial cement samples from Vietnam was collected and isolated for calcite precipitation bacteria. Out of ten total isolated strains, four strains were detected for the presence of crystals by observation in broth cultured medium (Figure 1B) and by using the light microscope. These strains were named N1 to N4 (Figure 2A). The colony appeared white, dry with calcite precipitation (Figure 1A).

The crystal was tested with diluted hydrochloric acid (HCl), all of four strains released tiny gas bubbles of carbonate (Figure 1C).

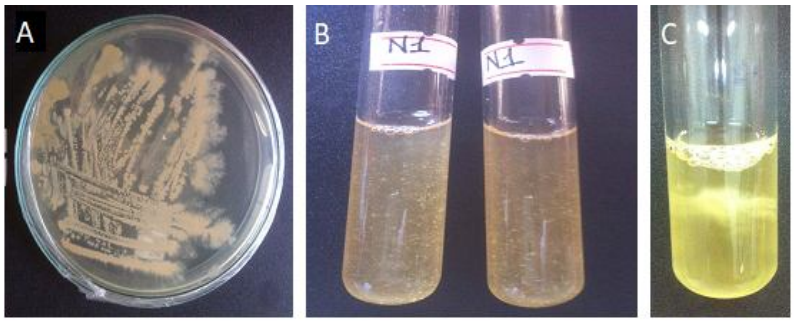

Figure 1. *Observation of calcite precipitation (A), on agar medium, (B), in broth medium, and (C), tiny carbonate gas released in the presence of HCl*

3.2. Microscope Phenotypic Characterization

Calcite precipitation bacterial strains were observed under microscope. We could observed the crystal under microscope when put a drop of liquid cultured medium on the slide. Gram stain and endospore stain were done. All of strains are Gram positive (purple/blue color), rod-shaped with single cells, but only N1 strain could form endospores (Figure 2).

Endospore are special resistant dormant structures formed within a cell that is capable of surviving in adverse environmental or hostile condition. They are extremely resistant to heat, desiccation, chemicals and radiation. [18]. By forming endospores, bacteria can withstand large mechanical stresses and chemically induced stresses during mixing of concrete and can remain viable for periods up to 200 years.

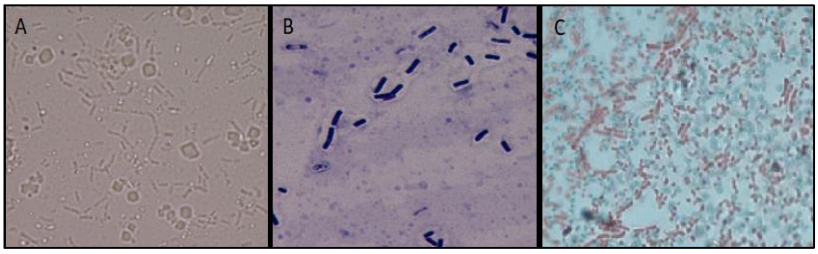

Figure 2. *Optical micrographs of isolated strain N1 under light microscope, (A), crystals and rod-sharped cells in a drop of broth cultured medium; (B), Gram (+) stain; and (C), endospore stain*

3.3. Calcium Carbonate Precipitation and Urease Activity

The urease activities were investigated. All four strains shown positive urease activity, urease enzyme turning the medium pink due to the pH sift to alkaline indicating the production of ammonia in the medium (Figure 3).

Figure 3. *Urease test of isolated strains; left, positive urease; right, negative urease*

Four isolated strains N1, N2, N3 and N4 were investigated the capability of inducing calcite precipitation. Strain N1 shown the formation more calcite than the others (Fig2). After 60h of incubation, the masses of the precipitates of four strains were 515±56 mg/l; 212±33 mg/l; 185±64 mg/l, 294±43 mg/l, respectively (Figure 4).

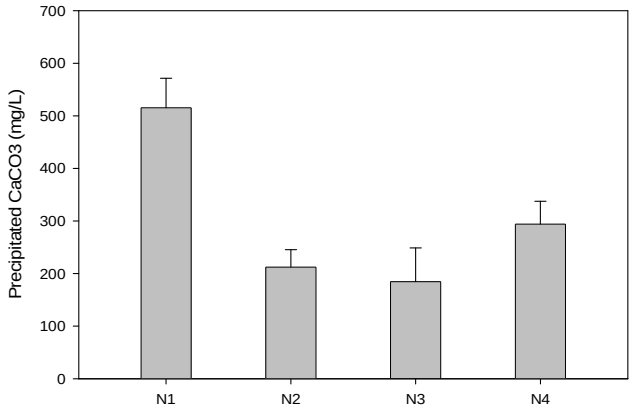

Figure 4. *Capability of calcite precipitation of four strains*

The results indicated that all four strains that isolated from cement samples has the same mechanism for the formation of calcite. The mechanism of urea degradation is widely studied and contributes to the maximum amount of calcite precipitated by the microorganisms [5-6]. This mechanism depends on the secretion and execution of the urease enzyme which results in the production of ammonia and carbonate. Ammonia release act to raise the pH of the medium which is a favorable condition for the precipitation of calcium carbonate. Carbonate binds calcium ions present in medium resulting in the formation of calcium carbonate crystals which was deposited in agar as well as in broth media. Similar results were reported for *Sporosarcina pasteurii* [19].

3.4. DNA extract, PCR amplification and sequencing

Due to the highest capability of calcite precipitation (up tu 500 mg/L) and the formation of endospore. The strain N1 have potential to survive long time in concrete structure for effective bio-calcification. This strain has been identified base on molecular characteristics. The 16S rDNA fragment from the genomic DNA was amplified by PCR reaction and then be sequenced. The sequence of this fragment was then analyzed by using the Blastx software (BLAST) (National Center for Biotechnology Information). 16S rDNA gene sequence analysis showed that there was a strong similarity (>99%) between strain N1 and representative strains in database of *Bacillus* spp. strains. Among that, the identification of N1 strain as *Bacillus thuringiensis* and *Bacillus wiedmaunii* were accepted with 100% (table 1). The aligned sequences of these strains were done further analysis by MegAlign pro (DNASTAR Lasergene 14) software. Distance matrix and phylogenetic tree results shown that N1 strain could be classified as *Bacillus thuringiensis.*

Table 1. *Sequences producing significant alignments**

Description	Max score	Total score	Querry cover	E-value	Ident	Accession
***Bacillus thuringiensis* strain YGD22-03, complete genome**	1626	19453	100%	0.0	100%	CP019230.1
***Bacillus wiedmannii* strain YT3 16S ribosomal RNA gene**	1626	1626	100%	0.0	100%	MF062955.1
***Bacillus* sp.strain BAB-6096 16S ribosomal RNA gene**	1622	1622	100%	0.0	99%	KY672905.1
***Bacillus cereus* strain MER_94 16S ribosomal RNA gene**	1622	1622	100%	0.0	99%	KT719669.1

*This table was constructed base on the data of NCBI Blast

We found that isolate and identified one bacterial strain N1- *Bacillus thuringiensis* that could form calcite precipitation. The mechanism to precipitate calcite of this strain is through the urea degradation.

4. Conclutions

Our experiments yielded 04 strains of cultivable bacteria that were able to produce urease. One of these strains shown the capability of the formation of endospore and high concentration of calcite (about 0.5g/l) through urease enzyme mechanism. This strain was identified as *Bacillus thuringiensis* base on 16S rDNA sequence. The results showed that ability of this strain to tolerate the extreme environment of cement, it could be used in remediating the cracks and fissures in various building or concrete structure. This is the first study in Vietnam to show the presence of at least one cultivable microorganism from cement sample capable of production of urease and precipitate calcite as the result of urea hydrolysis.

Acknowledgment: *This study was supported by Institute of Tropical Biology, Vietnam Academy of Science and Technology; and NTT Hi-Tech Institute, Nguyen Tat Thanh University.*

REFERENCES

[1] N.D. Belie, "Application of bacteria in concrete: a critical review," *RILEM Technical Letters*, 1, pp.56 – 6, 2016.

[2] A.M Neville, "Autogenous healing - A concrete miracle," *Concrete Int*, 2002, 24, pp.76-82.

[3] H. Knorre and W. Krumbein, *Bacterial calcification*. In Riding RE and Awramik SM (ed.). Springer-Verlag, Berlin, Germany, 2000, pp.25-31.

[4] F. Hammes, W.Verstraete, "Key role of pH and calcium metabolism in microbial carbonate precipitation," *Review Environmental Science Biotechnology*, 1, pp.3-7, 2002.

[5] M.B. Burbank, T.J. Weaver, B.C. Williams & R.L. Crawford, "Urease Activity of Ureolytic Bacteria Isolated from Six Soils in which Calcite was Precipitated by Indigenous Bacteria," *Geomicrobiology Journal*, 29, pp.389-395, 2012.

[6] K. L. Bachmeier, A. E. Williams, J. R. Warmington, and S. S. Bang, "Urease activity in microbiologically-induced calcite precipitation," *J. Biotechnol.*, 93, pp.171-181, 2002,.

[7] D.T Wright, A. Oren, "Nonphotosynthetic bacteria and the formation of carbonates and evaporites through time," *Geomicrobiol. J.*, 22, pp.27–53, 2005,.

[8] G.I Perez-Perez, C.B Gower, M.J Blaser, "Effects of cations on Helicobacter pylori urease activity, release and stability," *Infect Immun*, 62, pp.299–302, 1994.

[9] M.A Rivadeneyra, A. Ramos-Cormenzana, G. Delgado, R. Delgado, "Process of carbonate precipitation by Deleya halophila. Curr," *Microbiol*, 32, pp.308–313, 1996.

[10] M.A Rivadeneyra, G. Delgado, A. Ramos-Cormenzana, R. Delgado, "Biomineralization of carbonates by Halmonas eurihalina in solid and liquid media with different salinities: crystal formation sequence," *Res Microbiol*, 149, pp.277–287, 1998.

[11] K.B. Chekroun, C. Rodriguez-Navarro, M.T. Gonzalez-Munoz, J.M Arias, G. Cultrone, M. Rodriguez-Gallego, "Precipitation and growth morphology of calcium carbonate induced by Myxococcus xanthus: implications for recognition of bacterial carbonates," *J Sediment Res*, 74, pp.868–876, 2004.

[12] L. Chen, Y. Shen, A. Xie, B. Huang, R. Jia, R. Guo, W. Tang, "Bacteria-mediated synthesis of metal carbonate minerals with unusual morphologies and structures," *Crys Growth Des*, 9, pp.743–754, 2004.

[13] N.K. Dhami, M.S. Reddy, A. Mukherjee, "Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites," *J Microbiol Biotechnol*, 23, pp.707–714, 2013.

[14] C.H. Kang, J.H Choi, J.G Noh, D.Y Kwak, S.H Han, J.S So, "Microbially induced calcite precipitation-based sequestration of strontium by Sporosarcina pasteurii WJ-2.", Appl Biochem Biotechnol, 174, pp.2482–2491, 2014.

[15] M. Marvasi , K.L. Gallagher , L.C.Martinez , W.C.M.Pagan , R.E.R.Santiago, G.C.Vega & P.T.Visscher, "Importance of B4 Medium in Determining Organomineralization Potential of Bacterial Environmental Isolates," *Geomicrobiology Journal*, 29, pp.916-924, 2012.

[16] S.Wei, H.Cui, Z.Jiang, H. Liu, H. He, & N Fang, "Biomineralization processes of calcite induced by bacteria isolated from marine sediments," *Brazilian Journal of Microbiology*, 46(2), pp.455–464, 2015.

[17] J.F. MacFaddin, Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott, Williams & Wilkins, Baltimore, 2000.

[18] S. Krishnapriya, D.L. Venkatesh Babu, G.P. Arulraj, "Isolation and identification of bacteria to improve the strength of concrete," *Microbiological Research*, 174, pp.48-55, 2015.

[19] A. Hammad, F.N. Talkhan, A.E. Zoheir, "Urease activity and induction of calcium carbonate precipitation by Sporosarcina pasteurii NCIMB 8841," *J. Appl Sci Res*, 9 (3), pp.1525-1533, 2013.